

I claim:

1. A method for producing glycoproteins having carbohydrate structures similar to those produced by human cells in a lower eukaryote comprising  
providing a unicellular or multicellular fungal host, which does not express one or more enzymes involved in production of high mannose structures, and  
introducing into the host one or more enzymes for production of a carbohydrate structure selected from the group consisting of Man<sub>5</sub>GlcNAc<sub>2</sub>, Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>9</sub>GlcNAc<sub>2</sub>, wherein the enzymes are selected to have optimal activity at the pH of the location in the host where the carbohydrate structure is produced or which are targeted to a subcellular location in the host where enzyme will have optimal activity to produce the carbohydrate structure.
2. The method of claim 1 wherein the host is deficient in the activity of one or more enzymes selected from the group consisting of mannosyltransferases and phosphomannosyltransferases.
3. The method of claim 2 wherein the host does not express an enzyme selected from the group consisting of 1,6 mannosyltransferase, 1,3 mannosyltransferase, and 1,2 mannosyltransferase.
4. The method of claim 1 wherein the host is selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Saccharomyces cerevisiae*, *Saccharomyces sp.*, *Hansenula polymorpha*, *Kluyveromyces sp.*, *Candida albicans*, *Aspergillus nidulans*, and *Trichoderma reesei*.
5. The method of claim 2 wherein the host is an OCH1 mutant of *P. pastoris*.
6. The method of claim 1 comprising introducing into the host a nucleotide molecule encoding one or more mannosidases involved in the production of Man<sub>5</sub>GlcNAc<sub>2</sub> from Man<sub>8</sub>GlcNAc<sub>2</sub> or Man<sub>9</sub>GlcNAc<sub>2</sub>.
7. The method of claim 6 where the at least one mannosidase has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the mannosidase is localized, or having optimal activity at a pH between 5.1 and 8.0.

8. The method of claim 7 wherein the mannosidase enzyme has optimal activity at a pH between 5.9 and 7.5.

9. The method of claim 8 wherein the mannosidase enzyme is an  $\alpha$ -1,2-mannosidase derived from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, or *Bacillus* sp.

10. The method of claim 1 comprising providing a host that is able to form  $\text{Man}_5\text{GlcNAc}_2$  structures, displaying GnT I activity and having UDP-Gn transporter activity.

11. The method of claim 1 comprising providing a host which has a UDP specific diphosphatase activity.

12. The method of claim 1 comprising introducing into the host one or more enzymes selected from the group consisting of mannosidases, glycosyltransferases and glycosidases, wherein the enzymes are targeted to the endoplasmic reticulum, the early, medial, late Golgi or the trans Golgi network.

13. The method of claim 12 wherein the mannosidase enzyme is predominantly localized in the Golgi apparatus or the endoplasmic reticulum.

14. The method of claim 12 wherein the enzymes are localized by forming a fusion protein between a catalytic domain of the enzyme and a chimeric localization region encoded by at least one genetic construct formed by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof

15. The method of claim 14 comprising providing a chimeric localization region from an enzyme selected from the group of mannosyltransferases, diphosphotases, proteases, GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI, GalT, FT, and ST.

16. The method of claim 14 providing a catalytic domain encoding a glycosidase or glycosyltransferase selected from the group consisting of GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI, GalT, Fucosyltransferase and ST, that has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the enzyme is localized, or having optimal activity at a pH between 5.1 and 8.0.

17. The method of claim 1 comprising introducing into the host nucleotide molecules encoding one or more enzymes selected from the group of nucleoside sugar transporters consisting of UDP-GlcNAc transferase, UDP-galactosyltransferase, GDP-fucosyltransferase, CMP-sialyltransferase, UDP-GlcNAc transporter, UDP-galactose transporter, GDP-fucose transporter, CMP-sialic acid transporter, and nucleotide diphosphatases.

18. The method of claim 17 comprising genetically engineering the fungal strain to remove UDP or GDP by action of a diphosphatase.

19. The method of claim 1 wherein the glycoprotein includes *N*-glycans of which greater than 27 mole percent comprise fewer than six mannose residues.

20. The method of claim 1 wherein the glycoprotein comprises one or more sugars selected from the group consisting of galactose, sialic acid, and fucose.

21. The method of claim 1 wherein the glycoprotein comprises at least one oligosaccharide branch comprising the structure NeuNAc-Gal-GlcNAc-Man.

22. The method of claim 1 wherein the glycoprotein comprises *N*-glycans having fewer than four mannose residues.

23. The method of claim 1 wherein subsequent to isolation from the host, the glycoprotein is subjected to at least one further glycosylation or carboxylation reaction *in vitro*.

24. The method of claim 1 comprising the steps of

(a) providing a DNA library comprising at least two genes encoding exogenous glycosylation enzymes;

(b) transforming the host with the library to produce a genetically mixed population expressing at least two distinct exogenous glycosylation enzymes; and

(c) selecting from the population a host producing the desired glycosylation phenotype.

25. The method of claim 24 wherein the host is transformed two or more times with the library prior to the selection of a desired glycosylation phenotype.

26. The method of claim 24 wherein the library comprises at least one wild-type gene encoding a glycosylation enzyme.

27. The method of claim 24 wherein the library comprises at least one synthetic gene encoding a glycosylation enzyme.

28. The method of claim 24 wherein the library comprises at least one gene previously subjected to a technique selected from the list: gene shuffling, *in vitro* mutagenesis, and error-prone polymerase chain reaction.

29. The method of claim 24 wherein the library comprises at least one genetic construct formed by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof.

30. The method of claim 29 wherein a DNA fragment encodes an activity selected from the group consisting of mannosidase, UDP-GlcNAc transferase, UDP-galactosyltransferase, and CMP-sialyltransferase and the cellular targeting signal peptide is predominantly localizes the enzyme in an organelle selected from the group consisting of endoplasmic reticulum, cis Golgi, medial Golgi, and trans Golgi.

31. The method of claim 24 wherein the selection comprises the step of analyzing a glycosylated protein or isolated *N*-glycan by one or more methods selected from the group consisting of mass spectroscopy, liquid chromatography, characterizing cells using a fluorescence activated cell sorter, spectrophotometer, fluorimeter, or scintillation counter, exposing host cells to a lectin or antibody having a specific affinity for a desired oligosaccharide moiety, and exposing cells to a cytotoxic or radioactive molecule selected from the group consisting of sugars, antibodies, and lectins.

32. The host produced by the method of claim 1.

33. The glycoprotein produced by the method of claim 1.

34. The library of claim 24.